

1 Cross References to Other Applications

2 P This application is a Continuation-in-Part of my
3 application Serial No. 553,075, which in turn is a Continuation-
4 in-Part of each of my applications Serial Number 550,432, filed
5 February 18, 1975, now abandoned, entitled RECOGNINS AND THEIR
6 CHEMORECIPROCALs; Serial Number 450,404, filed March 12, 1974,
7 now abandoned; and Serial Number 385,451, filed August 3, 1973,
8 now abandoned.

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1 *P. 5411* THE INVENTION

2 This invention is directed to a novel group of com-
3 pounds, herein termed Recognins. Recognins are made by treating
4 tumor cells or artificial cancer cells and separating the
5 desired products. The Recognins may be used to prepare their
6 Chemoreciprocal, i.e., by contacting the Recognins for the
7 Recognins on a support with body fluids. These Chemoreciprocal
8 are useful for diagnostic and therapeutic purposes, i.e., for
9 diagnosing and treating cancers.

10 One of the Recognins of the present invention is
11 Astrocytin. Astrocytin is produced from brain tumor tissue,
12 preferably brain glioma tumor tissue. Protein fractions con-
13 taining the Astrocytin precursor are first extracted from the
14 tissue. A preferred method of accomplishing the extraction
15 is to treat the tissue with a neutral buffer under conditions
16 of homogenization or other techniques to disrupt the cells
17 and tissues in order to solubilize protein fractions which
18 contain the Astrocytin precursor.

19 At this point, the Astrocytin precursor is still
20 bound to many large molecular weight substances including
21 proteins, glycoproteins, lipoproteins, nucleic acids, nucleo-
22 proteins, etc. The solubilized proteins are then separated
23 from the resultant tissue extract. The extract solution from
24 the tissue is then clarified to remove insoluble particles.
25 The low molecular weight contaminants are then removed from
26 the resultant solution, by a preevaporation concentration
27 technique. The solution which is obtained is then treated to
28 cleave Astrocytin precursor from other contaminants in order
29 to obtain the protein fraction having a pK range between 1
30 and 4. Thus, for example, the solution is placed on a chroma-

1 tographic column and eluted with increasingly acidic solvents.
2 All of the fractions which are eluted in the neutral or acid
3 range down to pK 4 are discarded and those fractions with pK
4 range 1-4 are collected. The eluate is then treated to obtain
5 a product having a molecular weight of about 8,000. This is
6 accomplished, for example, by first filtering the material to
7 remove low molecular weight substances, i.e., those below
8 1,000 molecular weight, and filtering again to remove those
9 above 25,000. The fraction having a molecular weight between
10 1,000 and 25,000 is then further treated, i.e., by thin layer
11 gel (TLG) chromatography, to obtain Astrocytin.

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2/23/75 12 Thus Astrocytin may be produced by extracting brain
13 glioma tumor tissue with a neutral buffer, by repeated homo-
14 genization and high speed centrifugation, separating from
15 the resulting extract the fraction having a pK range of from
16 about 1 to 4, separating from said fraction the substances
17 having a high molecular weight, i.e., up to about 230,000,
18 and isolating therefrom the product Astrocytin having a molecu-
19 lar weight of about 8,000.

20 The product Astrocytin prepared in accordance with
21 this process is characterized by forming a single line precipi-
22 tate with its specific antibody in quantitative precipitin
23 tests and Ouchterlony gel diffusion tests, being soluble in
24 water and aqueous solutions having an acid or neutral pH, and
25 insoluble at an alkaline pH, having a spectrophotometric absorp-
26 tion peak wave length of 280 mμ and having a molecular weight
27 of about 8,000.

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2/23/75 28 Astrocytin is also characterized by having a very
29 high ^{percentage} ratio of residues of glutamic acid and aspartic acid and
30 a very high ratio of these acids to histidine. A further

1 analysis of Astrocytin is provided below.

2 In a manner similar to that described above, another
3 Recognin, called Malignin, is produced from artificial cancer
4 cells, i.e., cancer cells grown in vitro. Malignin has a
5 molecular weight of about 10,000 and similar but
6 distinct amino acid residue composition ^{to} ~~as~~ Astrocytin,
7 i.e., high ratios of glutamic acid and aspartic acid and high
8 ratios of these acids to histidine. A further analysis of
9 Malignin is provided below.

10 Thus, Malignin can be produced by extracting artificial
11 cancer cells grown in culture with a neutral buffer by repeated
12 homogenization and high speed centrifugation, separating from
13 the resulting extract the fraction having a pK range of about
14 1 to 4, separating from said fraction the substances having
15 a high molecular weight, i.e. up to about 230,000, and isolating
16 therefrom the product having a molecular weight of about 10,000.

17 Malignin prepared in accordance with this process
18 is characterized by forming a single line precipitate with its
19 specific antibody in quantitative precipitw tests and
20 Ouchterlony gel diffusion tests, being soluble in water and
21 aqueous solutions having an acid or neutral pH, and insoluble
22 at an alkaline pH, having a spectrophotometric absorption
23 peak wave length of 280 mμ and having a molecular weight of
24 about 10,000.

25 Recognins are further characterized by being capable
26 of complexing with bromoacetylcellulose to form bromoacetyl-
27 cellulose-Recognin and producing the specific antibodies Anti-
28 Recognin upon injection into mammals, said ^{Anti-Recognin being toxic to} producing fluor-
29 escence of glioma cells ^{in vitro} and
30 described in further detail below.

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1 Recognins, such as Astrocytin, Malignin and similar
2 substances are useful as products which may be introduced into
3 a biological system ~~body~~ to reduce foreign reactions, such
4 as by coating a material with a Recognin. A further example
5 may be to introduce a Recognin in order to produce the Chemo-
6 reciprocals in the biological system. They may also be used
7 nutritionally to encourage the growth of a particular biological
8 system of which they are a part. A further utility of Recognin
9 is the production of Target reagents which comprise the com-
10 plexes of the Recognin with a carrier to facilitate its appli-
11 cability in biological systems. Thus, for example, the complex
12 conveys the physical-chemical characteristics of the Recognin
13 itself. The carrier should be selected from those which form
14 a complex with the Recognin and which ^{are} ~~is~~ substantially bio-
15 logically inert.

16 Any substance known in the art which will form a
17 stable complex with polypeptides or proteins may be useful for
18 complexing with the Recognin. An example is a cellulose-based
19 material, such as bromoacetyl-cellulose. In addition to being
20 inert to the biological system, the carrier should be one that
21 does not alter the specific physical-chemical properties of
22 the Recognin which are useful for the purposes set forth herein.

23 The complexes of the Recognin and its carrier are
24 useful for producing, separating and identifying its chemo-
25 reciprocal in any biological system with which it is brought
26 into contact. The Recognin-carrier complex is also useful
27 for stimulating the production of its chemoreciprocal ^{Precursor} in any
28 biological system into which it is introduced.

29 One class of Chemoreciprocal are the anti-Recognins,
30 i.e. anti-Astrocytin and anti-Malignin. These may be made by

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1 injecting the Recognin into a biological system. An immuno-
2 logically effective dose of Recognin is brought into contact
3 with bodily ^{tissues or} fluids in a manner which induces an antibody
4 response in accordance with techniques known in the art for
5 producing antibodies. The anti-Recognins may be used for
6 the delivery of materials such as diagnostic, nutritional and
7 therapeutic agents to specific cells or sites in a biological
8 system which comprises introducing said agent in complexed form
9 with the anti-Recognin into the biological system. The anti-
10 Recognins are also useful for diagnosing the presence of tumor
11 cells in a histology section, by applying the Anti-Recognin
12 conjugated with a labeling substance such as dyes and radio-
13 active substances, to said section, whereby staining or radio-
14 active labeling occurs only with tumor cells. Yet another
15 use for anti-Recognins is for increasing the yield of other
16 useful Chemoreciprocal products (such as TAG, described below)
17 from a mammal which comprises injecting an immunologically
18 effective dose of ~~anti~~-Recognin into the mammal, or other bio-
19 logical system.

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20 Another class of Chemoreciprocal is Target reagents
21 complexed with their chemoreciprocal. For example, the
22 Target product of Astrocytin complexed with a carrier such
23 as bromoacetylcellulose is brought into contact with anti-
24 Astrocytin. This type of compound may be complexed with and
25 used for the delivery of diagnostic, nutritional and therapeutic
26 agents to specific cells or sites in a biological system.
27 These compounds may also be used for purification procedures.
28 For example, Anti-Astrocytin may be made by the decomplexing
29 of Bromoacetylcellulose-Astrocytin-Anti-Astrocytin by hydrolytic
30 treatment with an acid or proteinase enzyme. Target reagents

are also useful for increasing the amount of TAG products (described below) in a biological system, such as by bringing an immunologically effective dose of Target into contact with ^{tissues or} bodily fluids.

Additional Chemoreciprocal are TAG reagents (e.g. Target-Attaching Globulins). The TAG products are produced by bringing Target reagents into contact with body fluids for varying periods of time to form a complex and cleaving TAG therefrom. Two useful embodiments are S-TAG and F-TAG.

A process for producing S-TAG (Slow-Target-Attaching-Globulin) comprises reacting blood serum or other body fluid with Target (i.e. Bromoacetylcellulose-Malignin) for approximately two hours or more at a low temperature, e.g. about 4°C, and cleaving S-TAG from the resulting material, e.g. with dilute acid for approximately two hours at a temperature of about 37°C. The product S-TAG prepared in accordance with this process is characterized by being soluble in aqueous buffered solutions, forming a single line precipitate with its corresponding Recognin in Ouchterlony gel diffusion tests, being non-dialyzable in cellophane membranes, being retained by millipore filters which retain molecules over 25,000 molecular weight, having molecular weights in different states of aggregation as determined by thin layer gel chromatography of approximately 50,000, and multiplies thereof into the macro-globulin range and having a spectrophotometer absorption peak wave length of 280 mμ.

A process for producing F-TAG (Fast-Target-Attaching-Globulin) comprises reacting blood serum or other body fluid with Target (i.e. Bromoacetylcellulose-Malignin) for approximately 10 minutes at a low temperature, e.g. about 4°C, and

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1 cleaving F-TAG from the resulting material, e.g. with dilute
2 acid for approximately two hours at a temperature of about 37°C.
3 The product F-TAG prepared in accordance with this process is
4 characterized by being soluble in aqueous buffered solutions,
5 forming a single line precipitate with its corresponding
6 Recognin in Ouchterlony gel diffusion tests, being non-dialyzable
7 in cellophane membranes, being retained by millipore filters
8 which retain molecules over 25,000 molecular weight, having
9 molecular weights in different states of aggregation as deter-
10 mined by thin layer gel chromatography of approximately 50,000,
11 and multiples thereof into the macroglobulin range and having
12 a spectrophotometer absorption peak wave length of 280 mμ.

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13 TAG products are useful for detecting cancer tumors
14 in living mammals by determining the concentration of S-TAG
15 and F-TAG produced by a known volume of the mammal's blood
16 serum or other body fluid and correlating this concentration
17 with amounts determined to be indicative of cancer. TAG products
18 are also useful for diagnosing the presence of tumor cells in
19 a histology section, which comprises applying TAG conjugated
20 with a labeling substance such as dyes and radioactive sub-
21 stances, to said section, whereby staining or radioactive
22 labeling occurs only with tumor cells. TAG products additionally
23 have been found to be cytotoxic to tumor cells. TAG products
24 are also useful for directing the delivery of diagnostic,
25 nutritional and therapeutic agents to specific cells or sites
26 by introducing said agents in complexed form with the TAG
27 product.

1 Normal cell division in plants or animals is restricted
2 or inhibited when the cells come to occupy fully a particular
3 space. The mechanisms (a) by which normal cells "recognize"
4 that they have filled the space available to them, and (b) by
5 which the operation of this recognition mechanism in turn inhi-
6 bits cell division, have both been unknown. The inventor has
7 produced a group of compounds whose precursors are increased in
8 concentration when normal recognition and learning occur, and
9 which relate to recognition and learning in particles and cells,
10 and with the connection of cells to each other. These compounds
11 are termed RECOGNINS by the inventor. By attempting to produce
12 these compounds from normal cancer cells, the inventor has dis-
13 covered that they are absent as such, and that changes in their
14 molecular structure have occurred at the same time that the can-
15 cer cells have lost their ability (a) to recognize that they
16 have filled their normal volume, and/or (b) to stop dividing
17 when they have filled their normal volume.

18 The inventor has discovered novel compounds and methods
19 for producing such compounds. These new compounds are termed
20 RECOGNINS by the inventor. RECOGNINS are novel compounds which
21 have physicochemical characteristics which mimic those configura-
22 tions characteristic of cancer cells in terms of their failure
23 to ~~recognize~~ ^{recognize} and stop cell division. The use of RECOGNINS goes
24 ~~beyond~~ ^{beyond} ~~into~~ ^{into} the cancer mechanism, for immediate products
25 ~~and~~ ^{methods are} thereby provided which are useful in the diagno-
26 ~~sis and treatment~~ ^{sis and treatment} of cancer, and for its prevention.

27 ~~I have~~ ^{I have} discovered methods by which artificially cul-
28 ~~tured cells~~ ^{tured cells} can be used to produce MALIGNINS for the first time.
29 ~~The advantage~~ ^{The advantage} of the methods disclosed herein is that MALIGNINS
30 ~~and new~~ ^{and new} products from them can now be manufactured efficiently

1 in virtually limitless quantities.

2 This invention transcends the field of cancer research
3 and is immediately applicable to any and all biological systems
4 in which it is desired to influence all growth and metabolism.
5 Thus by the manufacture of the particular compound or compounds
6 of appropriate cell type in artificial culture, and the further
7 manufacture of products from these substances, specific influ-
8 ence may for the first time be brought to bear on any tissue,
9 cell, cell organelle, sub-organelle molecule or molecular aggre-
10 gate in any living system. Thus specific nutritional influences
11 at critical times in development, specific diagnostic, preventa-
12 tive and treatment methods, and the construction of artificial
13 bioelectrical systems (as in tissue or organ transplants) can all
14 be affected for the first time. These artificial bioelectrical
15 systems can now be made to bear the characteristics of the
16 specific RECOGNIN, MALIGNIN or their CHEMORECIPROCALs of the
17 normal tissue or component which they will neighbor and thus
18 avoid being "recognized" as "foreign" and thus avoid the reactions
19 to alien substances, including rejection.

20 Another aspect of this invention is the production of
21 a valuable specific antibody-like product (Anti-Astrocytin) to a
22 specific brain product (Astrocytin), permitting the use of this
23 antibody-like product to specifically complex with and, as a
24 specific delivery vehicle to, specific points in the nervous
25 system of all species. MALIGNINS and ASTROCYTIN are RECOGNINS.

26 Still another aspect of this invention is the produc-
27 tion from biological fluids of two new products, TARGET-
28 ATTACHING-GLOBULINS (TAG), which are so named because they are
29 produced by two reactions, the first reacting biological fluids
30 with a synthetic complex containing physicochemical configura-

1 tions which mimic those of the MALIGNINS and called TARGET, the
2 second, cleaving the specific TAG from the complex, and by the
3 measure of the TAG so produced obtaining a quantitative indica-
4 tion from the biological fluids of living organisms whether
5 there is present a tumor in that organism; hence a diagnostic
6 test for tumors. Because TAG products and ANTI-MALIGNIN are
7 physicochemically complimentary to MALIGNINS, they are termed
8 CHEMORECIPROCALs.

9 I have further discovered that two quantitatively and
10 qualitatively distinct TAG products can be produced depending
11 upon the time permitted for the reaction of serum with the
12 specific TARGET reagent used, and depending upon the time per-
13 mitted for the cleavage of the product which has been complexed.

14 After examining the amounts of these products which
15 could be produced from a number of different individuals with
16 brain tumors and various other medical disorders, as well as in
17 those with no apparent disease process, it became apparent that
18 the amounts of these two new products which could be produced
19 in a given individual was indicative of whether that individual
20 had a brain tumor, hence a serum diagnostic test for brain
21 tumors, the first to my knowledge.

22 The utility of these new products, in addition to
23 their use to diagnose from serum and other biological fluids the
24 presence of brain and other tumors, is illustrated by the demon-
25 stration that TAG and anti-RECOGNIN compounds ^{attach} to glial tumor
26 cells preferentially in histological sections of brain tumor and
27 surrounding tissue removed at surgery of the brain tumor. This
28 preferential labelling by TAG ^{and Anti-} RECOGNINS of tumor cells
29 is demonstrated through standard ^{immunofluorescent} fluorescent techniques.
30 Thus a new method is also available ^{later} for determining through

1 histological examination with a new degree of certainty whether
2 tumor cells are present in the tissue removed, and whether these
3 tumor cells have penetrated to the very edges of the tissue
4 removed indicating the likelihood that tumor still remains in
5 the brain or other organ, or that tumor cells are absent from
6 the periphery of the tissue removed, indicating the possibility
7 that all of the tumor has been removed from the brain or other
8 organ. In addition, TAG and Anti-RECOGNINS produced as described
9 have been found to be cytotoxic for glioma brain tumor cells
10 grown in tissue culture in vitro. This high affinity for tumor
11 cells in another medium, here grown in tissue culture, is further
12 evidence of the specific-coupling potential of the new product
13 TAG, and explains the adoption of the name TARGET-ATTACHING-
14 GLOBULINS (TAG) as do TAG's properties in regard to the synthetic
15 product TARGET, and to tumor cells in histological section.
16 Further, the cytotoxicity of TAG and anti-RECOGNINS for tumor
17 cells provides an additional new diagnostic test for serum of
18 patients who are suspected of suffering from a tumor. Thus, for
19 example, the serum or other body fluid of these patients is
20 reacted with TARGET to produce TAG and the product TAG is tested
21 in tissue culture growths of tumor cells for cytotoxicity. Both
22 the concentration of TAG and the degree of cytotoxicity manifes-
23 ted by the TAG which can be produced from a given individual's
24 serum may be not ^{only} ~~diagnostic~~ but also of value in tracing
25 the course of the ~~disorder~~ ^{disorder} preoperatively and postoperatively in
26 a given patient. ~~Loading~~ ^{new TAG} of radioactive and dye tracers to
27 TAG provides ~~the~~ ^{tumor} products which are useful in vivo in the
28 diagnosis of ~~tumor~~ ^{possibly} and in their exact localization. Thus the
29 injection of ~~radioactively~~ ^{radioactively} labelled TAG either intraarterially or
30 intravenously ~~into~~ ^{into} the cerebrospinal fluid, or directly into

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1 brain tissue or its cavities, permits the demonstration by radio-
2 active means, or by visualization of the coupled dye, of the
3 presence of a brain tumor, for it is only to the tumor cells
4 that the TAG specifically attaches. Further, this method permits
5 the precise visualization of the location of the brain tumor.
6 This can be seen to be an improvement of this in vivo diagnostic
7 method using anti-ASTROCYTIN produced in rabbit blood to label
8 the brain tumor, because the use of TAG produced from human
9 serum avoids the possibility of foreign protein reactions. Since
10 TAG and anti-RECOGNINS have the chemical specificity which per-
11 mits preferential attachment to ASTROCYTIN precursor containing
12 tumor cells both in vitro and in vivo, these products may also
13 be used therapeutically, as well as diagnostically, when coupled, e.g.,
14 with radioactive, proton capture agents, or other toxic physical
15 or chemical agents, so that these toxic substances may be local-
16 ized preferentially through these compounds' specificity of
17 attachment in the tumor cells as compared to their neighboring
18 normal cells. This selectivity is universally recognized as the
19 crucial, or at least one crucial factor for achieving effective
20 chemical or physical therapy of tumors, and a factor which has
21 hitherto not been achieved. Thus TAG has demonstrated efficacy
22 in attaching preferentially to the tumor cells, and should have
23 promise as a new therapeutic product for these reasons.

24 In the serum of patients with malignant tumors, as
25 will be seen in the examples below, one type of TAG, SLOW-TAG
26 (S-TAG) as distinguished from FAST-TAG (F-TAG), can be produced
27 in relatively greater amounts from a given volume of serum than
28 in patients without such tumors. This suggests that either one
29 of TAG's naturally occurring precursors (P-TAG) is increased in
30 concentration or that other factors exist which favor the rela-

1 tive in vitro production of S-TAG over F-TAG.

2 The possible relationship of the function of the
3 actual synthetic products TARGET and TAG ^{to their precursors, and in turn} to functions of postu-
4 lated but not demonstrated cell "antigens" and circulating
5 "antibodies" to them which may exist in vivo has yet to be eluci-
6 dated. Thus for example, in antibody-like fashion, F-TAG and
7 S-TAG produce single discrete lines of reaction with ASTROCYTIN
8 in Ouchterlony gel diffusion, and the injection of TARGET in
9 rabbits induces an increase in the yield of TAG products from

10 rabbit serum after reacting with TARGET. The finding that there
11 ^{may be} ~~is~~ a normal level of a ^{precursor} ~~substance~~ resembling circulating antibody

12 to a cell antigen which is hidden in the non-dividing cell
13 raises a question as to the possible function of the pair. It is

14 here proposed that TAG precursor (P-TAG) and TARGET-like sub-
15 stances exist in vivo which function in the control of cell
16 proliferation and cell death. Thus, for example, the exposure
17 of a cell constituent which normally is not directly exposed to
18 serum proteins may occur during cell division. The exposure of
19 this cell constituent could result in that constituent becoming
20 converted to a TARGET-like substance to which the attachment of

21 a P-TAG-like molecules from serum may then occur, which would
22 stimulate cell division or inhibit it. Alternatively, a non-
23 dividing cell which is injured or malfunctioning may expose a
24 TARGET-like substance to which the attachment of P-TAG-like
25 molecules may be reparative. However, under certain cell condi-

26 tions the attachment of P-TAG-like molecules may induce the
27 destruction of the cell (e.g. ANTI-GLIOMA-TAG synthetically pro-
28 duced as here described is markedly cytotoxic to glioma tumor

29 cells growing in tissue culture). This could ^{thus} represent a mirror of
30 a normal mechanism for the control of cell division, and ^{for} either

1 the repair or the removal of individual cells in the body
2 throughout the life of the organism. If the exposure of cell
3 constituents is abnormally increased so that abnormally large
4 amounts of cell TARGET-like substances are formed, as may
5 occur in rapidly dividing cancer cells such as in brain gliomas,
6 an increase in the concentration of one type of serum P-TAG re-
7 lative to another may be induced.

8 Whatever the actual function of the precursors, the
9 increase in the relative amount of predominately one type of TAG,
10 SLOW-TAG (S-TAG) which can be produced in vitro by the methods
11 here described from the serum of patients with malignant tumors
12 is the basis of the serum diagnostic test described in the
13 examples which follow.

DE The following examples illustrate the invention.

CL EXAMPLE I

P Production of Crude ASTROCYTIN-Precursor-Containing Fraction.

Human brain glioma tumor tissue, removed at surgery, is dissected free as possible of surface blood vessels and normal brain tissue. For a typical amount of dissected tumor tissue of 14 grams, the tissue is weighed into six 1.5 g. and two 1.0 g. aliquots. Each aliquot is then treated as follows.

Each aliquot is homogenized in neutral buffer solution by sonification or other mechanical means. For example, each aliquot is homogenized in 100 cc per g. of tissue of 0.005 M phosphate buffer solution, pH 7, in a Waring blender. Homogenization should be done in the cold to prevent denaturation of proteins. For example, the blender should be precooled in a cold room at 0-5°C and operated for about only three minutes.

The homogenate is then centrifuged for clarification, for example, at 80,000 times gravity for 30 minutes in a refrigerated ultracentrifuge. The soluble supernatant is decanted and kept in the cold. The insoluble residue is rehomogenized with a further 100 cc of neutral buffer and centrifuged as before, and the second soluble extract combined with the first. Best yields are obtained when this procedure of homogenization and centrifugation is repeated until less than 50 micrograms of protein per ml. of solution are obtained in the supernate. With most tissues this is accomplished by the fifth extraction.

The solutions thus obtained are combined and concentrated by preevaporation with subsequent dialysis, as by dialysis against 0.005 M phosphate buffer in the cold to produce a volume of 15 ml. The volume of this solution is noted, an aliquot is

1 taken for total protein analysis, and the remainder is fraction-
2 ated to obtain the protein fraction having a pK range between
3 1 and 4. The preferred method of fractionation is chromato-
4 graphy as follows.

5 The solution is fractionated in the cold room (4°C)
6 on a DEAE cellulose (Cellex-D) column 2.5 x 11.0 cm., which has
7 been equilibrated with 0.005 M sodium phosphate buffer. Stepwise
8 eluting solvent changes are made with the following solvents
9 (solutions): Solution (1) 4.04 g. NaH_2PO_4 and 6.50 g. Na_2HPO_4
10 are dissolved in 15 litres of distilled H_2O (0.005 molar, pH 7);
11 Solution (2) 8.57 g. NaH_2PO_4 is dissolved in 2480 ml. of distilled
12 H_2O ; Solution (3) 17.1 g. of NaH_2PO_4 is dissolved in 2480 ml. of
13 distilled H_2O , (0.05 molar, pH 4.7); Solution (4) 59.65 g. of
14 NaH_2PO_4 is dissolved in 2470 ml. distilled H_2O (0.175 molar);
15 Solution (5) 101.6 g. of NaH_2PO_4 is dissolved in 2455 ml. dis-
16 tilled H_2O (0.3 molar, pH 4.3); Solution (6) 340.2 g. of NaH_2PO_4
17 is dissolved in 2465 ml. of distilled H_2O (1.0 molar, pH 4.1);
18 Solution (7) 283.64 g. of 80% phosphoric acid (H_3PO_4) is made up
19 in 2460 ml. of distilled H_2O (1.0 molar, pH 1.0).

20 Add nervous tissue extract, 6 to 10 ml. volume. Let it
21 pass into column. Then overlay with Solution (1) and attach a
22 reservoir of 300 ml. of Solution (1) to drip by gravity onto the
23 column. Three ml. aliquots of effluent are collected by means of
24 an automatic fraction collector. The subsequent eluting solutions
25 are exchanged stepwise at the following elution tube numbers.
26 Solution (2): at tube 88, bring solution on column to top of
27 resin, then overlay and attach reservoir of 50 ml. of Solution
28 (2); Solution (2): at tube 98, bring solution on column to top
29 of resin, then overlay and attach reservoir of 75 ml. of Solution
30 (3); Solution (4): at tube 114, bring solution on column to top

(18)

1 of resin, then overlay and attach reservoir of 150 ml. of
2 Solution (4); Solution (5): at tube 155, bring solution on
3 column to top of resin, then overlay and attach reservoir of
4 125 ml. of Solution (5); Solution (6): at tube 187, bring solu-
5 tion on column to top of resin, then overlay and attach reservoir
6 of 175 ml. of Solution (7); continue eluting until at tube 260,
7 elution is complete. Use freshly prepared resin for every new
8 volume of tissue extract. Each effluent tube is quantitatively
9 analyzed for protein. The elutes in the tube numbers 212 to 230
10 are combined, and contain the crude products from which ASTROCYTIN
11 will be produced.

12 While data has been published on this crude material,
13 called fraction 10B in the past, [Protein Metabolism of the
14 Nervous System, pp. 555-69 (Pleum Press, 1970); Journal of
15 Neurosurgery, Vol. 33, pp. 281-286 (September, 1970)] the clea-
16 vage from fraction 10B of the specific product herein called
17 ASTROCYTIN has now been accomplished. Crude fraction 10B can
18 be prepared as a product in amounts between 0.1 and 10 mg. per
19 gm. of original fresh nervous system tissue from which it was
20 obtained. In addition to an ASTROCYTIN-precursor it contains
21 varying amounts of covalently bound carbohydrate residues includ-
22 ing a number of hexoses, namely glucose, galatose, mannose;
23 hexosamines, including glucosamine, galatosamine and mannosamine;
24 and occasionally other sugars, such as fucose, ribose and perhaps
25 rhamnose. It also contains large molecular weight protein pro-
26 ducts, several lipids and nucleic acids.

EXAMPLE 2

Production of Purified ASTROCYTIN from Crude ASTROCYTIN-Precursor-Containing Fraction.

The ASTROCYTIN-Precursor-Containing fraction is further isolated from contaminants. In the preferred embodiments, the material from Example 1 is chromatographed on Sephadex G-50 resin with a typical column of 40 cm. long, 2.5 cm. diameter, and 196 ml. volume. The pressure used is 40 mm. Hg.; the flow rate is 35 ml. per hour, and the buffer is 0.05 molar phosphate buffer solution, pH 7.2. The first (flow-through) peak contains ASTROCYTIN-Precursor together with impurities, whereas subsequent peaks contain only impurities.

In the preferred embodiment, the products in the above first flow-through peak are then concentrated on Sephadex G-15, then passed onto a column of Cellex-D with the same solutions, (1) through (7) as Example 1, and the same elution steps as performed in Example 1. The product ASTROCYTIN is present as a sharp peak in the same tubes (numbers 212-230) as before, thus maintaining its behaviour on Cellex-D chromatography without the presence of a large number of contaminants.

Low molecular weight contaminants may then be removed by techniques known to the art, such as millipore disc filtration. In the preferred method, the product ASTROCYTIN is freed of salt and other small molecular weight contaminants by filtration through Millipore Pellicon Disc No. 1000, 13 mm., which retains substances of molecular weight greater than 1000 and permits to pass through those of molecular weight less than 1000. The product ASTROCYTIN remains on the Pellicon Disc, and is recovered from it by washing with Solution (1) of Example 1.

ASTROCYTIN is then obtained by isolating the compound

1 having a molecular weight of about 8000 from the above solution.
2 A preferred method uses thin layer gel (TLG) chromatograph as
3 follows:

4 The apparatus used is the commercially available one
5 designed by Boehringer Mannheim GmbH; Pharmacia Fine Chemicals
6 and CAMAG (Switzerland). The resin 2.5 g. of Sephadex G-200
7 superfine is prepared in 85 ml. of 0.5 M. NaCl in 0.02 M.
8 $\text{Na}_2\text{HPO}_4\text{KH}_2\text{PO}_4$ Phosphate Buffer pH 6.8 (6.6-7.0). Allow to swell
9 two or three days at room temperature with occasional gentle
10 mixing. (Magnetic and other stirrers should not be used). The
11 swollen gel is stabilized for three weeks at refrigerator tem-
12 perature; however, bacterial and fungal growth may interfere with
13 the swollen gel. If the gel is to be kept for longer periods of
14 time, a small amount of a bacteriostatic agent should be added
15 (sodium Azide 0.02%) 2.5 g. of dry gel are used to make two
16 20 x 20 cm. glass plates of 0.5 mm. thick. The plates are
17 either allowed to dry at room temperature for 10 minutes and
18 transferred to a moist chamber where they can be stored for
19 about two weeks, or they are used immediately after appropriate
20 pre-equilibration. (Usually during the night for a minimum of
21 12 hours). The main function of equilibration is to normalize
22 the ratio between the stationary and mobile phase volumes. With
23 the pre-equilibrated plates in a horizontal position, substances
24 to be determined are applied with micropipettes as spots or as a
25 streak at the start line. 10 ml. to 20 ml. of 0.2-2% protein
26 solution is placed on the edge of a microscopic cover slide
27 (18 x 18 mm.) and held against the gel surface. In a few seconds
28 the solution will soak into the gel. All samples are first
29 prepared on the cover slides and then quickly applied. If not
30 enough material is used, it is difficult to locate individual

1 spots after separation. If too much material is applied no
2 defined separation occurs. The samples are diluted with buffer
3 for easier handling and the separation of samples is carried in
4 a descending technique with the plate at an angle of 22°. The
5 flow rate of about 1-2 cm/hour is most suitable. Marker sub-
6 stances (such as cytochrome C, haemoglobin, myoglobin or bromo-
7 phenol Blue labeled albumin) are applied at different positions
8 across the plate to give a check on possible variation of flow
9 across the plate and also to serve as reference proteins for
10 calculation of relative distance (mobility) of unknowns. After
11 application of samples, the plates are replaced in the apparatus
12 and the paper wick pushed slightly downwards to ensure good con-
13 tact with the gel layer. The paper wick must not drip. Excess
14 moisture is wiped off. The liquid solvent in the reservoir is
15 kept constant at 1 cm. from the upper end of the vessel. The
16 runs are usually completed in 4 to 7 hours depending on the pro-
17 gress of separation. With colored substances separation follows
18 directly. The separated spots of protein are easily made visible
19 by transferring them to a paper sheet replica of TLG plate after
20 the chromatographic separation has been completed, and by stain-
21 ing them on the prewashed methanol + H₂O + acetic acid -
22 90:5:5, for 48 hours. The paper sheet is 3 mm. filter paper. A
23 sheet of paper 20 x 18 cm. is placed over the gel layer and
24 pressed (rolled) just enough to ensure contact with the gel.
25 Care is taken not to trap air under the paper (replica) and not
26 to disturb the gel layer. The liquid phase is soaked off from
27 the gel layer by the paper and removed after about one minute,
28 immediately dried in an oven at a 60° temperature for 15 minutes
29 and stained in the normal way with any of the routine staining
30 procedures. Staining is performed by spraying the replica-paper

1 with 0.03% diazotized sulfanilic acid in 10% Sodium Carbonate
 2 (Pauley's Reagent). Staining can also be accomplished with a
 3 saturated solution of Amido Black in Methanol-Acetic Acid
 4 (90:10v/v is used); the staining time is 5-10 minutes. For de-
 5 staining, rinse with two volumes of the 90:10 methanol and acetic
 6 acid solution mixed with one volume of H₂O. It is difficult to
 7 obtain low background staining without very extensive washing.
 8 The plates themselves may also be dried at about 60°C (in an
 9 oven with air circulation) but only if the ASTROCYTIN is to be
 10 stained. For isolation purposes, the plate should only be air
 11 dried at room temperature. Over-heating can lead to cracking,
 12 but this can usually be avoided with at 50°-60°C temperature
 13 which dries a sephadex G-200 plate in 15-30 minutes. The dry
 14 plates are allowed to swell for 10 minutes in a mixture of
 15 methanol + H₂O + acetic acid (75:20:5) and stained in a saturated
 16 Amido Black in the same solvent system for five hours and subse-
 17 quently washed by bathing for two hours in the same solvent
 18 before they are dried. For molecular weight determinations the
 19 distance from the starting line to the middle of each zone is
 20 measured with an accuracy of 0.05 mm. either directly on the
 21 print (replica) or on the densitogram. The result is expressed
 22 by the R_m value defined as the ratio of the migration distance
 23 of the tested protein (d_p) to that of cytochrome C or myoglobin
 24 (d_m) which is used as the reference protein: Relating migration
 25 distance of tested substance to standard is the formula
 26 $(-R_m = \frac{d_p}{d_m})$. A straight calibration line is obtained by plotting
 27 the logarithm of the molecular weight of the standards
 28 used against the R_m . From this line the molecular weight of the
 29 unknown protein can be obtained. For most exact results six
 30 equal parts of the protein sample solution with standard, in this

case, Cytochrome C, before applying to the plate. By the above TLG procedure the product ASTROCYTIN is observed as a discrete spot at a distance of approximately 0.83 ± 0.02 with reference to the standard Cytochrome C, yielding an approximate molecular weight of 8000 for ASTROCYTIN. Several discrete products are separated in this procedure from ASTROCYTIN on the basis of slight differences in chemical structure and large differences in molecular weight. Thus, three products carried as contaminants to this point with molecular weight of approximately 64,000, 148,000 and 230,000, and one occasionally of molecular weight 32,000, have been detected and removed by the TLG methods described above. The product is ASTROCYTIN is aspirated with the gel in which it is contained, in dry form, dissolved in Solution (1) and freed of resin by centrifugation or other similar means.

The product ASTROCYTIN which has been produced at this stage is soluble in distilled water, soluble at neutral and acid pH, and insoluble at alkaline pH and has a spectrophotometric absorption peak wavelength of 280 m μ . It is a polypeptide with molecular weight, as stated above, of approximately 8000. Its covalently linked amino acids are shown by hydrolysis with 6N HCl then quantitative automatic determination to have the following average composition of amino acids:

| | Approximate Number of residues |
|---------------|-----------------------------------|
| Aspartic acid | 9 |
| Threonine | 5 |
| Serine | 6 |
| Glutamic acid | 13 |
| Proline | 4 |
| Glycine | 6 |

| | | |
|----|-------------------|----|
| 1 | Alanine | 9 |
| 2 | Valine | 4 |
| 3 | 1/2 Cystine | 2 |
| 4 | Methionine | 1 |
| 5 | Isoleucine | 2 |
| 6 | Leucine | 8 |
| 7 | Tyrosine | 2 |
| 8 | Phenylalanine | 3 |
| 9 | Lysine | 8 |
| 10 | Histidine | 2 |
| 11 | Arginine | 4 |
| 12 | | |
| 13 | Approximate Total | 88 |

14 Cysteic acid, hydroxyproline, norleucine, ammonia, isodesmosine,
 15 desmosine, hydroxylysine, lysinonorleucine and gamma-aminobutyric
 16 acid are all absent in detectable amounts, but a trace of gluco-
 17 samine may be present.

18 From 11 grams of the starting brain tumor tissue in
 19 EXAMPLE 1, approximately 3 mg. of purified ASTROCYTIN is produced
 20 by the above methods.

EXAMPLE 3

Production of MALIGNIN-Precursor in Artificial Cancer Cell Culture.

Generally, sterile technique is scrupulously maintained.

All solutions (e.g. Hank's Balanced Salt (BSS), F-10 Nutrient medium, fetal calf serum, trypsin solution) are incubated at about 35°C in a water bath for approximately 20 minutes or more before use.

Cells are removed from tumor tissue and grown in vitro for many generations using a suitable medium, such as described below. Pre-rinse beakers to be used with a sterilizing solution, for example 12-proponal plus Amphyl or creolin solution.

In the preferred embodiment, the artificial cancer cells (i.e., cells grown in vitro for many generations) are grown in 250 ml. flasks. The liquid medium in which the cells are growing is discharged into the pre-rinsed beakers. The cells are then washed gently with 5-10 ml. of Hank's BSS or other similar solution for about 30 seconds. Avoid agitation. All walls and surfaces are washed. The solution is clarified of cells by centrifugation in the cold from 10 minutes at 3,000 rpm. The medium is poured into a beaker as above. Add a small amount of buffered proteinase enzyme solution and rinse quickly to avoid digestion of the cells. In the preferred method, 1-2 ml. of trypsin solution (EDTA) is added and rinsed for only 10 seconds. Pour off the trypsin solution.

Add a similar volume of fresh trypsin ^{solution} and incubate until the cells are seen to be separated ^{from} the walls of the chamber through microscopic observation. ^{this} usually requires 5-10 minutes. Add a suitable growth ^{medium, such} as 50 ml. of a solution of 7-10 percent solution of fetal calf serum

1 in 100 ml. of F-10 Nutrient medium.

2 Twentyfive ml. of the fresh medium with cells is trans-
3 ferred to a new growth chamber for propagation and the remaining
4 25 ml. is kept in the first chamber for propagation. Both
5 chambers are placed in an incubator at 35°C for approximately
6 seven days. By the procedure of this Example to this point, an
7 artificial cancer cell culture is divided into two fresh cultures
8 approximately every seven days. This entire procedure may be
9 repeated as often as desired, at approximately seven-day inter-
10 vals, for each growth chamber. Thus, the number of ^{cells} ~~cultures~~
11 growing in vitro may be doubled approximately every seven days.

12 The cells may be extracted for the production of
13 MALIGNIN after approximately seven days of growth. For example,
14 cells growing in each 250 ml. growth chamber as described above,
15 may be recovered as follows.

16 The medium is transferred to a centrifuge tube and
17 centrifuged at 3,000 rpm in the cold for 10 minutes. The medium
18 is discarded. The cells remaining in the growth chamber are
19 scraped from the chamber walls and washed into the centrifuge
20 tubes with neutral buffer solution. The cells are washed twice
21 with neutral buffer solution, ^{centrifuged} ~~centrifuged~~ again at 3,000 rpm
22 in the cold, and the medium is ^{discarded} ~~discarded~~. The washed cells are
23 suspended in 10 ml. of neutral phosphate buffer until ready for
24 extraction of crude MALIGNIN-^{Precursor-Containing} ~~containing~~ fraction.
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87 CL EXAMPLE 4

1 Production of Crude MALIGNIN-Precursor-Containing
2 Fraction.
3

4 Washed cells suspended in neutral buffer from
5 EXAMPLE 3 are mechanically disrupted under conditions which avoid
6 denaturation of most proteins. In the preferred method, the
7 washed cells are treated in the cold with a sonifier for 20
8 seconds.

9 After sonification the cell residues are centrifuged
10 at 30,000 rpm for 30 minutes and the supernatant decanted. Ten
11 ml. aliquots of buffer solution are used to wash remaining cells
12 from the chamber and these are added to the remaining cell
13 residues. Sonify and centrifuge as above and combine the super-
14 natants. Repeat the process once more.

15 The combined supernatant is perevaporated to reduce the
16 approximate 30 ml. volume to about 6-7 ml. An aliquot is taken
17 for total protein analysis and the remainder is fractionated
18 according to the methods of EXAMPLE 1 for ASTROCYTIN Precursor.
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EXAMPLE 5

Production of Purified MALIGNIN Product from Crude MALIGNIN-containing Fraction.

The product MALIGNIN is further isolated from contaminants by the methods of EXAMPLE 2 for ASTROCYTIN.

In the TLG step of the preferred embodiment, the product MALIGNIN is observed as a discrete spot at a distance of approximately 0.91 ± 0.02 with reference to the standard cytochrome C, yielding an approximate molecular weight of 10,000 for MALIGNIN.

The product MALIGNIN which has been produced at this stage is soluble in distilled water, soluble at neutral or acid pH, and insoluble at alkaline pH and having a spectrophotometric absorption peak of 280 mu. It is a polypeptide with molecular weight of approximately 10,000. Its covalently linked amino acids are shown by hydrolysis with 6N HCl then quantitative determination to have the following average composition of amino acids:

Approximate Number
of Residues

| | |
|---------------|----|
| Aspartic Acid | 9 |
| Threonine | 5 |
| Serine | 5 |
| Glutamic Acid | 13 |
| Proline | 4 |
| Glycine | 6 |
| Alanine | 7 |
| Valine | 6 |
| 1/2 Cystine | 1 |
| Methionine | 2 |

| | | |
|---|-------------------|----|
| 1 | Isoleucine | 4 |
| 2 | Leucine | 8 |
| 3 | Tyrosine | 3 |
| 4 | Phenylalanine | 3 |
| 5 | Lysine | 6 |
| 6 | Histidine | 2 |
| 7 | Arginine | 5 |
| 8 | | — |
| 9 | Approximate Total | 89 |

10 the amino acids cysteic acid, hydroxyproline, norleucine,
11 ammonia, isodesmosine, desmosine, hydroxylysine, lysinonorleucine
12 and gamma-aminobutyric acid being absent in detectable amounts.
13 A typical yield of pure MALIGNIN from twelve 250 ml.
14 reaction chambers of EXAMPLE 3 together is approximately 1 mg.
15 of MALIGNIN.

CL
P
EXAMPLE 6

Production of TARGET Reagents from RECOGNINS.

ASTROCYTIN, prepared as in EXAMPLE 2 above, or
MALIGNIN, prepared as in EXAMPLE 5 above, is complexed with a
carrier to produce TARGET reagent.

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In the preferred embodiment, ASTROCYTIN or MALIGNIN is
dissolved in 0.15 M NaH_2PO_4 - citrate buffer, pH 4.0. A
bromoacetyl-resin, for example bromoacetylcellulose (BAC) having
1.0 to 1.5 milliequivalents Br per gram of cellulose, stored in
the cold, is prepared in 0.15 M NaH_2PO_4 buffer, pH 7.2. Convert
the buffer to pH 4 by pouring off the pH 7.2 buffer solution and
adding 0.15 M NaH_2PO_4 - citrate buffer, pH 4.0. The ASTROCYTIN
or MALIGNIN solution and the BAC solution are stirred together
(10:1 BAC to RECOGNIN ratio) for 30 hours at room temperature,
then centrifuged.

It is preferred that all sites on the BAC which are
available to bind to RECOGNIN be bound. This may be accomplished
as follows. The supernatant from the immediately preceding step
is lyophilized and the protein content determined to indicate
the amount of ASTROCYTIN or MALIGNIN not yet complexed to BAC.
The complexed BAC-ASTROCYTIN (or BAC-MALIGNIN) is
resuspended in 0.1 M bicarbonate buffer pH 8.9, stirred 24 hours
at 4° to permit the formation of chemical bonds between the BAC
and the ASTROCYTIN or MALIGNIN. After the 24 hours, the suspen-
sion is centrifuged and supernatant analyzed for protein. The
complexed BAC-ASTROCYTIN or BAC-MALIGNIN is now resuspended in
0.05 M aminoethanol - 0.1 M bicarbonate buffer pH 8.9 in order
to block any unreacted bromine. The suspension is centrifuged,
and the supernatant is kept but not analysed because of the pre-
sence of aminoethanol. Removal of all unbound ASTROCYTIN or

1 MALIGNIN is then accomplished by centrifugation and resuspension
2 for three washings in 0.15 M NaCl until no absorbance is measured
3 on the spectrophotometer at 266 mμ. The BAC-ASTROCYTIN or BAC-
4 MALIGNIN complex is now stirred in 8 M urea for 2 hours at 38°C,
5 centrifuged, then washed (three times usually suffices) with 8 M
6 urea until no absorbance is shown in the washings at 266 mμ. The
7 complex is then washed with 0.15 M NaCl two times to rid of urea.
8 The complex is then stirred at 37°C in 0.25 M. acetic acid for
9 2 hours to demonstrate its stability. Centrifuge and read super-
10 natant at 266 mμ ~~no~~ ^{8.5} absorbance should be present. This
11 chemically complexed BAC-ASTROCYTIN or BAC-MALIGNIN is therefore
12 stable and can now be used as a reagent in the methods described
13 below; in this stable reagent form it is referred to as TARGET
14 (TOPOGRAPHIC-ANTIGEN-LIKE-REAGENT-TEMPLATE) because it is a syn-
15 thetically produced complex whose physical and chemical properties
16 mimic the stable cell-bound precursor of ASTROCYTIN or MALIGNIN
17 when it is in a potential reactive state with serum components.
18 For storing, TARGET reagent is centrifuged and washed until
19 neutralised with 0.15 M NaH₂PO₄ buffer pH 7.2.

20 TARGET reagents may be prepared from bromoacetyl
21 liganded carriers other than cellulose, such as bromoacetylated
22 resins or even filter paper.

EXAMPLE 7

Production of antisera to Astrocytin, Malignin and TARGET.

Antisera to Astrocytin, Malignin or TARGET reagents may be produced by inducing an antibody response in a mammal to them. The following procedure has been found to be satisfactory.

One mg. of RECOGNIN (Astrocytin or Malignin) is injected into the toe pads of white male rabbits with standard Freund's adjuvant, and then the same injection is made intraperitoneally one week later, again intraperitoneally ten days and, if necessary, three weeks later. Specific antibodies may be detected in the blood serum of these rabbits as early as one week to ten days after the first injection. The same procedure is followed for TARGET antigen by injecting that amount of TARGET which contains 1 mg. of Astrocytin or Malignin as determined by Folin-Lowry determination of protein.

The specific antibody to Astrocytin is named Anti-Astrocytin. The specific antibody to Malignin is named Anti-Malignin. Similarly, the specific antibody to TARGET reagent is named Anti-Target.

These antibodies show clearly on standard Ouchterlony gel diffusion tests for antigen-antibody reactions with specific single sharp reaction lines produced with their specific antigen.

The presence of specific antibodies in serum can also be tested by the standard quantitative precipitin test for antigen-antibody reactions. Good quantitative precipitin curves are obtained and the micrograms of specific antibody can be calculated therefrom.

Further evidence of the presence of specific antibodies in serum can be obtained by absorption of the specific antibody

1 Anti-Astrocytin onto Bromoacetyl-cellulose-Astrocytin (BAC-
2 Astrocytin) prepared above. The antiserum containing specific
3 Anti-Astrocytin can be reacted with BAC-Astrocytin. When the
4 serum is passed over BAC-Astrocytin only the specific antibodies
5 to Astrocytin bind to their specific antigen Astrocytin. Since
6 Astrocytin is covalently bound to Bromoacetyl-cellulose the
7 specific antibody, Anti-Astrocytin, is now bound to BAC-
8 Astrocytin to produce BAC-Astrocytin-Anti-Astrocytin (BACA-Anti-
9 Astrocytin). This is proved by testing the remainder of the
10 serum which is washed free from BAC-Astrocytin. On standard
11 Ouchterlony diffusion no antibodies now remain in the serum which
12 will react with Astrocytin. It is therefore concluded that all
13 specific antibodies (Anti-Astrocytin) previously shown to be
14 present in the serum, have been absorbed to BAC-Astrocytin.
15 Furthermore, when Anti-Astrocytin is released from its binding
16 to BAC-Astrocytin it is thereby isolated free of all contaminat-
17 ing antibodies. This release of Anti-Astrocytin may be accom-
18 plished by washing the BACA-Anti-Astrocytin complex with 0.25 M
19 acetic acid (4°C., 2 hrs.) which has been shown above not to
20 break the BAC-Astrocytin bond.

21 Still further evidence of the presence of specific
22 antibodies in serum can be obtained by adsorption of the specific
23 antibody Anti-Malignin onto Bromoacetyl-cellulose-Malignin (BAC-
24 Malignin) prepared above. The antiserum containing specific
25 Anti-Malignin can be reacted with BAC-Malignin. When the serum
26 is passed over BAC-Malignin only the specific antibodies to
27 Malignin bind to their specific antigen Malignin. Since Malignin
28 is covalently bound to Bromoacetyl-cellulose the specific anti-
29 body, Anti-Malignin, is now bound to BAC-Malignin to produce
30 BAC-Malignin-Anti-Malignin (BACM-Anti-Malignin). This is proved-

1 by testing the remainder of the serum which is washed free from
2 BAC-Malignin. On standard Ouchterlony diffusion no antibodies
3 now remain in the serum which will react with Malignin. It is
4 therefore concluded that all specific antibodies (Anti-Malignin)
5 previously shown to be present in the serum, have been absorbed
6 to BAC-Malignin. Furthermore, when Anti-Malignin is released
7 from its binding to BAC-Malignin it is thereby isolated free of
8 all contaminating antibodies. This release of Anti-Malignin may
9 be accomplished by washing the BACM-Anti-Malignin complex with
10 0.25 M acetic acid (4°C., 2 hrs.) which has been shown above not
11 to break the BAC-Malignin bond.

12 The antibodies to TARGET show clearly on standard
13 Ouchterlony gel diffusion tests for antigen-antibody reactions
14 with specific single reaction lines produced with TARGET which
15 show a line of identity with the line of reaction to ANTI-
16 ASTROCYTIN or ANTI-MALIGNIN antisera (i.e. that produced to the
17 injection of ASTROCYTIN or MALIGNIN themselves). Some rabbits,
18 it has been noted, have levels of ANTI-TARGET in their blood
19 prior to being injected with TARGET. these ANTI-TARGET sub-
20 stances, when reacted specifically with TARGET reagent as to be
21 described in tests of human sera, lead to the production of
22 approximately equivalent amounts of the two types of TAG, S-TAG
23 and F-TAG (see later EXAMPLES).

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EXAMPLE 8

Detection of Malignant Tumors by Quantitative Production in vitro of TARGET-ATTACHING-GLOBULINS (TAG) from Biological Fluids.

TARGET reagent prepared in accordance with EXAMPLE 6 is washed to remove any unbound RECOGNIN which may be present due to deterioration. The following procedure is satisfactory. TARGET reagent is stirred for two hours at 37°C. with acetic acid, centrifuged, the supernatant decanted, and the optical density of the supernatant read at 266 mμ. If there is any absorbance, this wash is repeated until no further material is solubilized. The TARGET is then resuspended in phosphate buffered saline, pH 7.2. (Standard S-TAG and F-TAG purified from previous reactions of human serum by the procedure described below can be used if available, as reference standards to test the TARGET reagent, as can whole rabbit serum which has been determined to contain S-TAG and F-TAG by other TARGET preparations).

The Slow-Binding (S-TAG) determination is performed as follows: Frozen serum stored more than a few days should not be used. Serum is carefully prepared from freshly obtained whole blood or other body fluid by standard procedures in the art. The following procedure has been found to be satisfactory. Blood is allowed to clot by standing for 2 hours at room temperature in a glass test tube. The clots are separated from the walls with a glass stirring rod, and the blood allowed to stand at 4°C for a minimum of 2 hours (or overnight). The clots are separated from the serum by centrifuging at 20,000 rpm at 4°C for 45 minutes. The serum is decanted into a centrifuge tube and centrifuged again at 2000 rpm at 4°C for 45 minutes. The serum is decanted and a 1% Solution of Methiolate (1g. in 95 ml. water and

1 5 ml. 0.2 M bicarbonate buffer pH 10) is added to the extent of
2 1% of the volume of serum.

3 Serum samples, prepared by the above or other procedures,
4 of 0.2 ml. each are added to each of 0.25 ml. aliquots of TARGET
5 suspension reagent containing 100-200 micrograms of RECOGNIN per
6 0.25 ml. TARGET reagent, in duplicate determination. The sus-
7 pension is mixed at 4°C in a manner to avoid pellet formation.
8 For example, a small rubber cap rapid shaken may be used for 1-2
9 seconds and then, with the tubes slightly slanted, they may be
10 shaken in a Thomas shaker for about 2 hours or more. The TARGET
11 reagent and protein bound to it are separated from the serum. ^{the}
12 ^{which} procedure has been found to be satisfactory is the following. The
13 tubes are then centrifuged at 2000 rpm for 20 minutes at 4°C, the
14 supernatant decanted, the pellet which is formed by centrifugation
15 washed 3 times by remixing and shaking at room temperature with
16 0.2-0.3 ml. of 0.15 M Saline, centrifuged and the supernatants
17 discarded.

18 The protein which remains attached to the TARGET is cleave
19 therefrom and quantitatively determined. For example, 0.2 ml. of
20 0.25 M acetic acid is added, the suspension shaken for 1 to 2
21 seconds with a rubber cup shaker, then in a Thomas shaker for about
22 2 hours in a 37°C incubator. The tubes are centrifuged at 2000
23 rpm at 4°C for 30 minutes. The supernatant is carefully decanted
24 to avoid transferring particles and the optical density of the supe
25 natant is read at 280 mu. The value of the optical density is
26 divided by a factor of 1.46 for results in micrograms per ml. seru
27 protein (S-TAG). Duplicate determinations should not vary more
28 than 5%. Any other procedure effective for determining protein
29 content may be used, such as Folin-Lowry ^{Lowry} determination, but
30 standards must be specified to determine the range of control and

1 tumor values of S-TAG minus F-TAG concentration.

2 The Fast-Binding (F-TAG) determination is performed as
3 follows: Frozen serum stored more than a few days should not be
4 used. Serum is carefully prepared from freshly obtained whole blood
5 or other body fluid by standard procedures in the art. The pro-
6 cedure given above in this EXAMPLE for serum preparation is
7 satisfactory.

8 Serum samples, prepared by the above or other procedures
9 are allowed to stand at 4°C for 10 minutes less than the total time
10 the S-TAG serum determinations were allowed to be in contact with
11 TARGET reagent above [e.g. 1 hour 50 minutes if a "two hour"
12 S-TAG determination was made]. This procedure equilibrates the
13 temperature histories of S-TAG and F-TAG determinations.

14 Add 0.2 ml. samples of the temperature equilibrated
15 serum to each of 0.25 ml. aliquots of TARGET suspension reagent
16 containing 100-200 micrograms of RECOGNIN per 0.25 ml. TARGET
17 reagent, in duplicate determination. The suspension is then
18 mixed at 4°C for approximately 10 minutes in a manner to avoid
19 pellet formation. For example, a small rubber cap rapid shaken
20 may be used for 1-2 seconds and then, with the tubes slightly
21 slanted, they may be shaken in a Thomas shaker for approximately 10
22 minutes. The TARGET reagent and protein bound to it are separ-
23 ated from the serum. ^{One of the} procedures ^{which} has been found to be satis-
24 factory is the following. The tubes are then centrifuged at 2000
25 rpm for 20 minutes at 4°C, the supernatant decanted, the pellet
26 which is formed by centrifugation washed 3 times by remixing and
27 shaking at room temperature with 0.2-0.3 ml. of 0.15 M Saline,
28 centrifuged and the supernatants discarded.

29 The protein which remains attached to the TARGET is
30 cleaved therefrom and quantitatively determined. The procedure

described above in this EXAMPLE for determining S-TAG concentration is satisfactory. Any other procedure effective for determining protein content may be used, such as Folin-Lowry determination, but standards must be specified to determine the range of control and tumor values of S-TAG minus F-TAG concentration.

4/24/75
The final results are expressed as TAG micrograms per ml. of Serum, and equal S-TAG minus F-TAG. TAG values in non-brain tumor patients and other controls currently range from zero (or a negative number) to 140 micrograms per ml. of serum. TAG values in brain tumor patients currently range from 141 to 500 micrograms per ml. of serum. In the first "blind" study of 50 blood samples conducted according to the procedures of this EXAMPLE utilizing TARGET reagent prepared from Astrocytin and bromoacetylcellulose, 11 of 11 brain tumors and 28 of 32 normals were correctly identified. One of the 4 supposed normals (i.e., non-brain tumor controls) turned out to have a cancer of the thyroid gland which had apparently been successfully treated some years before. The three remaining normals were individuals aged 60-70 who were in poor health, possibly having nondiagnosed cancer. Of the remaining 7 samples, three out of three cases of Hodgkin's Disease were correctly identified; one sample in the tumor range (141-500 ug. TAG/ml.) corresponded to a patient having a severe gliosis, and three samples in the non-tumor range (0-140 ug. TAG/ml.) corresponded to patients having respectively, an intracranial mass diagnosis uncertain but non-tumor, and osteosarcoma (non-brain tumor) and a melanotic sarcoma (non-brain tumor).
8/23/75
8/24/75

A subsequent study conducting according to the procedures of this example utilizing TARGET reagent prepared from MALIGNIN and bromoacetylcellulose correctly identified three out of three malignant brain tumors and all normals.

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2/13/15

81/ Diagnosis of Tumor Cells by Immunofluorescence

1 * The compounds Anti-Astrocytin, Anti-Malignin, and S-TAG
2 have been shown to attach preferentially to tumor cells. This
3 specificity permits use of these compounds to diagnose tumor cells
4 in histology sections by conjugating dyes or radioactive substances
5 to Anti-Astrocytin, Anti-Malignin, or S-TAG. Standard labeling
6 techniques may then be used. A procedure using S-TAG is as follows.

7 One procedure which has been found satisfactory is a
8 modified St. Marie procedure. Human brain tumor specimens are
9 frozen and 5 micron thick sections cut. These are stored in a
10 moist container at minus 70°C for 4 to 8 weeks before staining.
11 The conjugate may be a standard antiserum such as goat anti-rabbit
12 conjugate. The conjugate is labeled by techniques known in the
13 art with fluorescein or other labeling substance. Fluorescein
14 labeled goat anti-rabbit conjugate as commercially available may be
15 used. The fluorescent technique used was a standard one in which
16 a 1:200 to 1:400 solution of TAG is incubated for about 30 minutes
17 or more on the tumor section, followed by washes to remove un-
18 attached TAG. Three washes with phosphate buffered saline has been
19 found satisfactory. Conjugate incubation with fluorescein-labeled
20 conjugate followed by washes is then performed, followed by micro-
21 scopic inspection. Normal cells and their processes fail to stain
22 both in tumor sections and in control sections of normal non-tumor
23 brain. Fluorescence is brightly present in tumor glial cells and
24 their processes.

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With Fluorescent Signal From TAG

1 The uses of TAG products coupled with a signal emitter such
2 as a dye or a radioactive label to detect cancer cells is described
3 for example, at pages 7, 9, 12, 13 and 14 herein. In this
4 EXAMPLE 9A, the detection of non-brain malignant cells is
5 described.

6 As described in EXAMPLE 8 utilizing human serum; in the
7 determination of TAG, after the anti-malignin antibody was bound
8 to the immobilized antigen and non-bound serum proteins washed
9 away, the antibody was cloven from the binding with 0.25 M acetic
10 acid at 37°C for 2 hours and the TARGET reagent separated from it
11 by centrifugation. The TAG antibody solution was quantitated by
12 means of its absorption at 280 mμ. The TAG solutions were stored
13 at -20°C, then thawed and combined, brought to pH 7 by titration
14 with 6N NaOH, dialyzed against phosphate buffered saline pH 7,
15 filtered and concentrated on Millipore Pellicon 1000 membranes,
16 centrifuged to clear insoluble protein and the immune globulin
17 complexes concentrated and freed of immunologically non-active
18 compounds by Cellex D and Blue Sepharose CL6B (Pharmacia) chroma-
19 tography. This human anti-malignin antibody reacts with anti-
20 human gamma globulin in Ouchterlony double diffusion. When TAG
21 is used with fluorescein conjugated to anti-human gamma globulin
22 in standard double layer Coons immunofluorescence it stains malig-
23 nant glia, breast carcinoma, ovarian carcinoma, adenocarcinoma of
24 colon, and other types of cancer cells in postoperative and biopsy
25 tissue sections, as well as in human sputum, bronchial washings,
26 pleural effusion fluid, gastric aspirate and bladder urine. The
27 concentration of protein in TAG which yield clear fluorescence
28 when controls are negative, is 1 to 10 μg per section.

29 The production of a "purified" TAG was undertaken by
30 reacting the sera from patients with a variety of cancers with

bromoacetylcellulose - MALIGNIN by methods earlier described
(EXAMPLE 8). The antibody bound in this reaction was cleaved
with 0.25 M acetic acid, quantified by measurement at O.D.
280 using a conversion factor of 1.46 for gamma globulin,

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31 1 frozen and stored at -20°C. This antibody was found to
2 contain immunoglobulin as determined by anti-human gamma-globulin
3 antiserum specific for gamma chains (BioRad Laboratories, Inc.)
4 and with anti-FAB and anti-Fc fragments (Miles Laboratories).
5 It also reacts with rabbit anti-human albumin (BioRad
6 Laboratories).

7 It was found that whereas 10 to 50 micrograms
8 of protein TAG are required to produce specific immuno-
9 fluorescent staining of cells which contain Malignin,
10 only 1 to 10 micrograms of purified protein TAG are required
11 for this specific staining in all sections, and in a few,
12 less than one microgram has been found to suffice.

13 It was found that the most active preparation of
14 purified TAG is that which is eluted with the highest ionic
15 strength elution, i.e., from 0.15 M to 1.5 M. Any method
16 of production which uses this fact is useful; three
17 preferred methods are given below.

18 P₃ Method I P₃ Fractionation of TAG by chromatography
19 with DEAE cellulose (Cellex D, BioRad Laboratories) was
20 first employed with step-wise elution with increasing
21 ionic strength and decreasing pH, the same sequence of
22 eluants as that given in Example I for the production
23 of Crude Astrocytin-Precursor-Containing Fraction. Good
24 separation was obtained of the bulk of the protein into
25 three fractions, Peak I obtained with Solution 1 (see
26 Example 1) and Peak II obtained with Solution 6 and
27 Solution 7. Ouchterlony double diffusion showed the
28 TAG in Peak I still to contain appreciable protein with
29 albumin mobility, and while Peak II contained most of the
30 albumin, appreciable IgG could be detected. Rechromatography

1 of Peak I gave a progressively pure IgG until, after the
2 seventh chromatography, essentially no albumin (less than
3 3%) could be detected by Ouchterlony gel diffusion in
4 which 5 to 10 micrograms of human albumin was detectable
5 with rabbit anti-human albumin. The IgG so obtained was
6 prone to denaturation and loss of immunological reactivity
7 after a few days standing at 0-5°C.

8 Method II P A second fractionation of TAG was made
9 with chromatography on Sepharose CL-6B (Pharmacia, Inc.)
10 starting with low molarity buffer (0.005 M phosphate) and
11 proceeding in two steps of 0.15 M and 1.5 M to elute the
12 balance of the protein. As with the Cellex D, one
13 passage was found to be inadequate to separate, and recycling
14 slowly improved the product. Once again, the most active
15 fraction vis-a-vis anti-malignin antibody was in the 1.5 M
16 fraction.

17 Method III P Chromatography with Sepharose CL-6B
18 next to the glass fritted disc and Cellex D layered above
19 the Sepharose proved to be the most satisfactory method.

20 The graphical representation in Fig. 1 shows the
21 fractions obtained on chromatography of TAG utilizing
22 Method III. After the first eluate of 200 mls., 50 ml.
23 or smaller sub-fractions were collected. The protein
24 content of each eluate was determined by the optical
25 density at 280 m μ with a uniform factor of 1.46 based
26 on gamma-globulin used to convert to micrograms for
27 calculating recoveries. The absolute amount of protein
28 requires correction in those fractions in which there is
29 appreciable albumin. The points at which the stepwise
30 solvent changes were made are indicated by arrows. The

1 subfractions are designated by Roman Numerals I through VIII.

2 The solvents corresponding to letters A-F at
3 the arrows were as follows:

4 A - 0.01 M TRIS (pH 7.2)

5 B - 0.05 M TRIS with 0.1M NaCl (pH 7.2)

6 C - PBS*, 0.11 M NaCl (pH 7.2)

7 D - PBS, 0.165 M NaCl (pH 7.2)

8 E - PBS, 0.33 M NaCl (pH 7.2)

9 F - 0.05 M TRIS, 1.5 M NaCl (pH 7.2)

10 In the following Table are shown the recoveries
11 from each fraction, a semi-quantitative determination in
12 each of the gamma-globulin and albumin in each, as well
13 as the activity of each fraction in the immunofluorescent
14 staining of cancer cells. (The plus sign indicates reaction,
15 zero no reaction and plus/minus reaction in some cases).

16 *PBS = Phosphate Buffered Saline

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TOAGGX

TABLE

| Fraction | I | II | III | IV | V | VI | VII | VIII |
|--|---------------|--------------|---------------|---------------|--------------|--------------|--------------|---------------|
| Recovered ug % | 2,877 12.5 | 1,140 5.0 | 2,351 10.2 | 2,942 12.8 | 1,808 7.9 | 2,230 9.7 | 2,125 9.3 | 7,477 32.6 |
| Immunodiffusion Against: Anti-human IgG, specific for gamma chains | +++ | ++ | ++ | + | o | + | ++ | +++ |
| Anti-human albumin | + | + | + | ++ | +++ | +++ | +++ | +++ |
| Anti-Fab | + | ++ | o | ++ | + | o | ++ | +++ |
| Anti-Fc | ++ | ++ | + | ++ | + | o | + | +++ |
| Immunofluorescence | + | + | + | + | + | + | + | +++ |

Photographs were prepared

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Fig. 2 is a photograph showing the line of reaction between anti-human gamma-globulin specific for gamma chains for each of Fractions I and II from above ~~(left and below in photograph)~~.

Photographs were taken

[^]
Figs. 3a and 3b are photographs showing the use of TAG (Fraction VIII from above) to stain non-brain malignant cells. ^{1. 22} Fig. 3a is a stain of bronchogenic carcinoma cells in the bronchial washings of a patient ^{and} Fig. 3b is a stain of lymphoma cells in the pleural fluid of a patient.

Non-cancer cells do not fluoresce. The TAG (1 to 10 ug in 0.1 ml phosphate buffered saline (PBS)) is applied to the surface of packed cells on a glass slide incubated 30 minutes, washed three times with PBS and then layered with fluorescein-conjugated anti-human IgG diluted until non-malignant control tissues give essentially no fluorescence. The cells are visualized with a Zeiss fluorescent microscope using a tungsten lamp and filters BG 23, BG 12 and 500.

(47)

EXAMPLE 9B

Detection of Cancer Cells With
Radioisotope Signal From Tag

In this Example, the feasibility of attaching a radioactive label to TAG is demonstrated. Second, the injection into animals of this radio-labeled TAG has been accomplished and shown to be safe and effective. Third, the radio-labeled TAG localized preferentially in the cancer tissue when compared to normal tissue, thus indicating that the specificity previously demonstrated in vitro of the preference for cancer cells which is conveyed by the use of specific anti-Malignin TAG products is confirmed in vivo.

The Labeling of TAG with ^{99m}Tc Technetium (^{99m}Tc)

Procedure for Labeling

1. Two preparations of TAG were used, here designated TAG-1 and TAG-2. TAG-1 and TAG-2 (concentration of each 0.4 mg/0.5ml) were added to separate sterile evacuated vials.
2. To each vial was added 0.1 ml of a stannous chloride solution (10 mg $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ in 100 ml of 0.01 N HCl). The vials were mixed for 3-4 minutes.
3. 0.1 ml. (6mCi) of ^{99m}Tc -pertechnetate (sodium salt) was added and mixed 2-3 minutes.

Procedure for determining labeling efficiency

Samples of the ^{99m}Tc -TAG-1 and ^{99m}Tc -TAG-2 were tested for labeling efficiency by descending paper chromatography using Watman No. 1 paper with 85% methanol as the solvent. A similar study was done with Sodium Pertechnetate- ^{99m}Tc which acted as a control.

After 2 hours, the papers were removed from the chromatography tank and divided in two sections: (1) 1 cm about the origin; (2) the remaining paper up to the solvent front. Each section was then counted in a gamma well scintillation counter and its content of radioactivity determined (cpm).

Approximately 50 lambda were plated on each paper strip.

Procedure for Antigen-Antibody Reaction

A portion of the labeled solution was also plated on an Ouchterlony gel plate to determine its ability to react with malignin in the antigen-antibody reaction. After a 3 hour period, the resulting sharp reactive lines were removed from the gel and their content of radioactivity measured. An equal portion of the gel not involved in the reaction was also removed and its content of radio activity was also measured as background.

Results

Labeling Efficiency

TABLE 1 - Labeling Efficiency of ^{99m}Tc -TAG-1 and ^{99m}Tc -TAG-2

| COMPOUND | SITE ON PAPER | CPM | % | CHEMICAL SPECIES |
|--------------------------------------|---------------|--------------------|--------|--------------------------|
| NaTcO_4 - ^{99m}Tc | origin | 4.94×10^5 | 7.33% | reduced TcO_4^- |
| NaTcO_4 - ^{99m}Tc | solvent front | 6.25×10^6 | 92.67% | TcO_4^- |
| TAG-1 | origin | 4.35×10^6 | 98.47% | TAG- ^{99m}Tc |
| TAG-1 | solvent front | 6.76×10^4 | 1.53% | TcO_4^- |
| TAG-2 | origin | 1.96×10^6 | 98.01% | TAG- ^{99m}Tc |
| TAG-2 | solvent front | 3.98×10^4 | 1.99% | TcO_4^- |

TABLE 2 - ANTIGEN-ANTIBODY RESECTION

| GEL AREA | COUNTS PER MIN | % |
|----------------|--------------------|--------|
| TAG-2 line | 1.99×10^6 | 92.04% |
| Background gel | 1.72×10^5 | 7.96% |

Conclusions

The following conclusions were reached relative to the quality control tests employed:

1. ^{99m}Tc -pertechnetate was reduced by stannous chloride to a more reactive oxidation state (+4+5).
2. The reduced pertechnetate labeled both the TAG-1 and TAG-2 preparations.
3. The ^{99m}Tc -TAG-2 was tested for its ability to retain its activity and was found to retain its ability to react immunologically.

The Use of Radio-Labeled TAG in vivo to Detect Cancer Cells

Wistar rats were injected intracerebrally with C18 glioma tumor cells which had had previous passages in rats and in tissue culture. The rats were observed for the first signs of growing tumor, such as weakness, tremor or unsteadiness. These symptoms first appear seven to 10 days from injection, and with fast growing tumors result in death within three to four days in many animals, and one week in all. As soon as symptoms appeared, the animals were injected with labeled TAG intravenously in the tail vein, then the animal anesthetized at varying times, the brain removed, the tumor dissected free of normal brain, and the radioactivity in each dissected specimen compared.

Preliminary ^{99m}Tc -TAG experiment

| Animal | Sacrifice (hr. post injection) | Tumor wt., mg. | Counts/gm/min. | |
|--------|-----------------------------------|-------------------|----------------|-----------------|
| | | | Tumor | Normal Brain |
| A | 1.25 | 1.9 | 149,100 | 13,400 |
| B | 5.30 | 6.0 | 16,200 | 6,600 |
| C | 7.21 | 23.0 | 53,000 | 5,800 |
| D | 24.10 | 29.0 | 66,700 | 7,500 |

Tumor and normal brain specimens were counted overnight in the gamma-well counter. All samples and standards were decay corrected for convenience to the mid-count of the first sample in the sequence.

Conclusion

The preferential localization of radioactivity in tumor as compared to normal tissue is demonstrated above.

(51)

1 Demonstration that Anti-Astrocytin, Anti-Malignin and S-TAG are
2 Cytotoxic to Tumor Cells Growing in Tissue Culture.

3 Standard tests for determining cytotoxicity may be used.
4 Generally, the number of cells in a fixed counting chamber, usually
5 arranged to contain about 100 live cells, is counted. These cells
6 are then treated with the agent being tested and the number of
7 cells which are still alive is counted.

8 In a standard test of cytotoxicity of S-TAG Solution ob-
9 tained in accordance with the methods of EXAMPLE 8 against cells
10 in tissue culture derived from a patient with a glioblastoma Grade
11 III-IV, well characterized as of glial origin, S-TAG produced
12 death of all cells in the counting chamber even when in high dilution
13 of 1:100 and 1:1000, representing as little as 0.2 and 0.02 ug. of
14 S-TAG per ml. of solution. Similar results are obtained with high
15 dilutions of Anti-Astrocytin and Anti-Malignin.

16 Both the specificity exhibited in EXAMPLE 9 and the
17 cytotoxicity demonstrated in EXAMPLE 10 are highly relevant to
18 the therapeutic possibilities of Anti-Astrocytin, Anti-Malignin and
19 S-TAG for brain tumors in man. While these therapeutic uses are in
20 the future, the practical diagnostic potential of both of these
21 phenomena for tumor tissue removed at operation but requiring diag-
22 nosis by histology is already demonstrated herein.

Hydrolytic Cleavage of RECOGNINS

A solution of RECOGNIN, in this case either Astrocytin or Malignin at pH between 1 and 2 is allowed to stand in the cold. After 7 to 14 days, TLG chromatography shows the product to have been reduced in molecular weight by approximately 200. When the solution is allowed to stand longer, further units of approximately 200 molecular weight are cleaved every 7 to 10 days. Thus with Astrocytin the molecular weight is reduced from 8,000, and with MALIGNIN the molecular weight is reduced from 10,000, in each case by units of approximately 200 sequentially.

The physicochemical specificities of Astrocytin are retained by each product down to approximately 4,000 molecular weight. The physicochemical specificities of Malignin are retained by each product down to approximately 5,000 molecular weight. This is shown by Ouchterlony gel diffusion tests against Anti-Astrocytin and Anti-Malignin, respectively.

This cleavage can also be accomplished enzymatically, as with trypsin and other proteinases, with similar results.

The molecular weights of these compounds prepared by hydrolytic cleavage of RECOGNINS may be approximately defined by the following formulae:

For products having the physicochemical specificities of Astrocytin; $4000 + 200 \times = Y$

For products having the physicochemical specificities of Malignin; $5000 + 200 \times = Y$

wherein Y is the molecular weight of the product and X is an integer from 0 to 19.

EXAMPLE 12

1 Production of Artificial Tissue or Organ with RECOGNINS

2 A rigid walled tube of plastic, metal, or other suitable
3 rigid material is dipped in or impregnated with a highly concen-
4 trated, [i.e., 10 mg./ml.] ^{viscous solution of} ~~viscous solution of~~ RECOGNIN, in this
5 case either Astrocytin or Malignin, until all surfaces are fully
6 coated with the RECOGNIN. Alternately, RECOGNIN solution is passed
7 through and around the tube under pressure until all surfaces are
8 fully coated. The tube is then dried in air or in vacuo, or
9 lyophilized. The process of coating is repeated several times in
10 order to build up multiple molecular layers of RECOGNIN coating.

11 The tube is now ready to be placed in a cavity or in a
12 tissue which contains Astrocytin or Malignin-like precursors in the
13 neighboring tissue or fluid of a living mammal. This artificial
14 tissue or organ may be used to minimize or eliminate reaction ^{which} ~~as~~
15 foreign substances without RECOGNIN coating would ~~incite~~.

16 Artificial tissues or organs of other geometries may
17 similarly be produced.